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Characterization of a salt-tolerant aminopeptidase from marine *Bacillus licheniformis* SWJS33 that improves hydrolysis and debittering efficiency for soy protein isolate

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ABSTRACT

An aminopeptidase was isolated from the marine *Bacillus licheniformis* SWJS33 (BLAP) and purified. According to the tandem mass spectrometry, the enzyme displayed 11% amino acid identity with the aminopeptidase from *Bacillus* (gi|496687392). BLAP exhibited maximum activity at 60 °C and pH 8.0–8.5 and had a molecular mass of 100 kDa. The presence of NaCl enabled 50% improvement of enzyme activity with 10–15% NaCl being the best. The observed inactivation by EDTA and bestatin and activation by Co^{2+} and Ag^{+} indicated that the obtained enzyme was a metalloaminopeptidase. Such an aminopeptidase could further improve the hydrolysis degree of soy protein isolate hydrolysates catalyzed by papain, Alcalase 2.4 L or Flavourzyme 500MG from 8.5%, 9.5% or 14.4–18.8%, 18.7% or 20.1%, respectively, while decreasing the bitter intensity score of the SPI hydrolysates catalyzed by Alcalase 2.4 L from 3.6 to 0.4.

1. Introduction

Controlled proteolysis of various protein-rich raw materials using proteases to produce protein hydrolysates with desirable bioactivity and flavors has become an important and sustainable approach to add value to local bioresources. Discovering proteases with high efficiency and novel characteristics at low costs from the nature such as marine system, to enrich the existing list of commercial preparations, is one of the strategies for the enzyme industry. Such a demand motivates this current study.

There exist various commercial proteases and most of which are endoprotease except for some in the form of mixtures of endoprotease and exoprotease such as Flavourzyme. Endoproteases have demonstrated high efficiency towards the hydrolysis of aquatic products and animal proteins but unsatisfactory outcomes (including poor protein recovery, low hydrolysis degree and unpleasant taste) for plant proteins such as soybean, wheat gluten and peanut proteins (Chen, Chen, Ren, & Zhao, 2011; Tchorbanov, Marinova, & Grozeva, 2011). Exoproteases attack the peptide chain at the ends (i.e. the N- or C-terminus of a protein) through removing a single amino acid or sometimes a di-/tri-peptide. These enzymes play important roles in improving hydrolysis efficiency as part of the preprocessing steps besides physical methods (Chen et al., 2011), and modifying the flavor of protein-rich products through debittering and monitoring flavor development. For example, aminopeptidases had been used to remove hydrophobic amino acids from the N-terminus of polypeptides (Huang et al., 2015; Li, Lin, Chen, Fu, & Wu, 2015; Stressler, Eisele, Schlayer, Lutz-Wahl, & Fischer, 2013; Wang et al., 2011) and in cheese ripening (Wilkinson, Guinee, O'Callaghan, & Fox, 1994). Though the introduction of aminopeptidases could obviously improve the hydrolysis efficiency, its large scale application is not well realized due to the limited species of commercial aminopeptidase.

Aminopeptidases (EC 3.4.11.) are an important member of exoprotease family, which can catalyze the cleavage of peptide bond linking the terminal amino acid at the free N-terminal end of a polypeptide chain. They can be classified according to their origin, location, preference for specific amino-terminal amino acid substrates, sensitivity to inhibitors, or different requirements for divalent metal ions (Taylor, 1993). Among the existing plant, animal and microorganism sources, microorganisms currently represent the most promising and reliable source for obtaining novel enzymes such as aminopeptidases due to its biochemical diversity, easy cultivation and other advantages such as relatively high

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stability and activity associated with their unique life habitats in the marine environment involving high salinity, high pressure, low temperature and special lighting conditions (Bull, Ward, & Goodfellow, 2000; Stach, Maldonado, Ward, Goodfellow, & Bull, 2003).

The microorganisms that had been reported to produce aminopeptidase were mainly *Aspergillus* (Matsushita-Morita et al., 2010), *Streptomyces* (Wu et al., 2010), *Pseudomonas* (Wu, Zhou, Zhou, Gao, & Tian, 2014), *Lactic acid bacteria* (Tchorbanov et al., 2011) and *Bacillus* (Rodríguez-Absi & Prescott, 1978) genus. *Bacillus licheniformis* is an important industrial microorganism because of its generally regarded as safe (GRAS) status, high growth rate and broad substrates (Voigt et al., 2004). Its ability to produce numerous desirable hydrolytic enzymes, especially alkaline serine protease (subtilisins; annual pure enzyme output: ~500 metric tones) further makes this strain industrially important (Schallmey, Singh, & Ward, 2004). However, only a few studies had applied *Bacillus licheniformis* to produce aminopeptidase, e.g. Rodriguez-Absi et al. purified an extracellular aminopeptidase from *Bacillus licheniformis* in 1978 for hydrolyzing dipeptides, aminoacylnaphthylamides, and amino acid amides (Rodríguez-Absi & Prescott, 1978), Pavlova et al. isolated an aminopeptidase from the thermophilic strain of *Bacillus licheniformis* for splitting off the N-terminal leucin in short peptides and hydrolyzing leucinamide (Pavlova, Rotanova, & Zholner, 1988).

In this present study, we aimed at isolating and purifying an aminopeptidase from marine-derived *Bacillus licheniformis* SWJS33. The obtained enzyme was evaluated through standard characterization and its application in plant protein hydrolysis.

2. Materials and methods

2.1. Materials and chemicals

Bacillus licheniformis SWJS33 was newly isolated from the deep-sea mud of South China Sea (kindly provided by South China Sea Institute of Oceanology Chinese Academy of Science) and deposited in the China General Microbiological Culture Collection Center (CGMCC No.7388). DEAE-Sepharose Fast Flow and Superdex 200 pg were purchased from GE Healthcare (Beijing, China). L-Leucine-*p*-nitroaniline (Leu-*p*NA), Glu-*p*NA, Lys-*p*NA, Pro-*p*NA, Met-*p*NA, bovine serum albumin (BSA), β-mercaptoethanol (β-ME), dithiothreitol (DTT), SDS, bestatin hydrochloride, phenylmethanesulphonyl fluoride (PMSF), ethylenediaminetetra-acetic acid (EDTA), pepstatin and soybean trypsin inhibitor (STI) were from Sigma (Beijing, China). Phe-*p*NA, Ala-*p*NA and Arg-*p*NA were from GL Biochem (Shanghai, China). Media components were purchased from Guangzhou Huankai Microbial Technology Co., Ltd. (Guangzhou, China). Soy protein isolate (SPI) was obtained from Guangzhou Honsea Co., Ltd. (Guangzhou, China). Alcalase 2.4 L and Flavourzyme 500MG were purchased from Novozymes (food grade; Beijing, China). Papain was obtained from Baiao Biochemistry Co., Ltd. (food grade; Jiangmen, China). All the other chemicals and solvents were of analytical grade.

2.2. Preparation of aminopeptidase

To produce aminopeptidase, the strain *Bacillus licheniformis* SWJS33 was grown in a medium (pH 7.0) consisting of glycerin 3.0 g, glucose 5.0 g, yeast extract 10.0 g, KH_2PO_4 0.5 g, $MgSO_4$ 0.3 g, $(NH_4)_2SO_4$ 1.0 g, $CaCl_2$, 1.0 g, sea salt 10.0 g and distilled water 1.0 L. The culture was incubated at 37 °C with constant shaking at 150 rpm for 48 h to facilitate fermentation. The fermented mixture was then centrifuged at 10,000*g* and 4 °C for 10 min in a CR22G high-speed centrifuge (Hitachi Co., Tokyo, Japan). The supernatant was collected as crude *Bacillus licheniformis* SWJS33 aminopeptidase (BLAP). The fermentation of BLAP was performed in duplicate.

2.3. Analysis of aminopeptidase activity and protein concentration

The leucine aminopeptidase activity was quantified following the method of Tan et al. (Tan & Konings, 1990) with some modifications. An aliquot (80 μL) of the diluted sample by Tris-HCl buffer (50 mM, pH 8.5) was mixed with 20 μL of Leu-*p*NA (20 mM). The mixture was subject to heating at 40 °C for 10 min and the reaction was stopped by adding 100 μL glacial acetic acid. A blank was set up through mixing 100 μL glacial acetic acid with the same sample before the substrate addition. The absorbance at 405 nm was measured. One unit of enzyme activity (LAP mL^{-1}) was defined as the amount of enzyme that liberates 1 μmol of leucine-*p*-nitroanilide per minute. Standard curve was plotted using 4-nitroaniline.

The protein concentration was measured based on the Coomassie method using brilliant blue G-250 to bind protein in order to measure the absorbance peak shift (from 465 to 595 nm), as well as using BSA as a known protein standard (Lowry, Rosebrough, Farr, & Randall, 1951). Appropriate dilutions of the test sample were performed.

2.4. Aminopeptidase purification

The crude BLAP was precipitated with ammonium sulfate using the fraction at 60% concentration according to the preliminary experiments. The resultant precipitate was collected after centrifugation (10,000*g*, 4 °C, 20 min) and re-suspended in 20 mM Tris-HCl buffer (pH 8.5). Then it was transferred into dialysis bags (MWCO: 10 kDa) and dialysed against the same Tris-HCl buffer at 4 °C for 48 h (the buffer was changed every 12 h). The dialysed solution was lyophilized (R2L-100KPS, Kyowa Vacuum Engineering, Tokyo, Japan) and stored in a desiccator over silica gel at 4 °C for further use. BLAP purified at this process was usually regarded as food grade enzyme.

Further purification of the dialyzed enzyme was performed using the AKTA pure system (GE Healthcare, USA) fitted with DEAE-Sepharose Fast Flow column (2.6 × 20 cm). The lyophilized enzyme (200 mg) was re-suspended in 4 mL of 20 mM Tris-HCl buffer (pH 8.5) and loaded to the pre-equilibrated column and the system was washed with increasing concentrations of NaCl solutions (0.1, 0.2, 0.3, 0.4 and 0.5 M; in Tris-HCl buffer) at a flow rate of 10 mL/min. Each corresponding eluate fraction (5 mL) was collected. The fractions containing BLAP activity were collected and further loaded onto a Superdex 200 pg column (1.6 × 60 cm). Then elution using the 20 mM Tris-HCl buffer (pH 8.5) was carried out at a flow rate of 1.0 mL/min. Each fraction with 4 mL of eluate was collected and the resulting enzyme was desalted by dialysis and then lyophilized (the enzyme termed "purified BLAP"). Molecular weight of the purified BLAP was analyzed following Laemmli (Laemmli, 1970) using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and 12% acrylamide under reducing conditions. After electrophoresis, the gels were stained with Coomassie brilliant blue R-250.

2.5. Aminopeptidase identification by tandem mass spectrometry

The purified BLAP in the SDS-PAGE was subject to digestion with trypsin (sequencing grade, Promega). The digested peptides were then analyzed on an ABI 4800-plus MALDI-TOF/TOF mass spectrometer (Applied Biosystems Foster City, USA) in the Institute of Life and Health Engineering, Jinan University. The MS spectra were analyzed and the peptide sequence was determined using the Mascot Program and the NCBI BLAST online search tool.